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ESTRUTURA GENÉTICA ESPACIAL DE UMA POPULAÇÃO DE BAMBUS EM HOTSPOT DE BIODIVERSIDADE

SPATIAL GENETIC STRUCTURE OF A POPULATION OF BAMBOOS IN A BIODIVERSITY HOTSPOT

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RESUMO

A diversidade genética é um dos três níveis de organização da biodiversidade, sendo a força motriz por trás do surgimento e adaptação das espécies a diferentes habitats. A interação entre a variabilidade genética presente no seio de uma população e elementos do ambiente onde esta ocorre eventualmente se traduzirá sob a forma de uma estrutura genética espacial, i.e., a distribuição espacial não-aleatória dos genótipos. Informações acerca da estrutura genética espacial de uma população constituem uma valiosa ferramenta para o estudo de populações e também da história evolutiva de uma espécie. O objetivo do presente trabalho foi investigar como a estrutura genética de uma população está organizada através de diferentes escalas espaciais, e de que maneira essas escalas relacionam-se entre si. Para tanto, escolhemos como modelo de estudo o bambu lenhoso *Merostachys neesii* Rupr. (Poaceae: Bambusideae), uma espécie endêmica da Mata Atlântica cujo ciclo de vida e características da história de vida as tornam um excelente modelo para estudos sobre estrutura genética espacial. No primeiro capítulo, descrevemos os marcadores moleculares utilizados nas análises genéticas dos capítulos subsequentes. No segundo capítulo, analisamos a estrutura genética espacial de uma população de *M. neesii* localizada na Floresta Ombrófila Densa Montana no Parque Estadual da Serra do Mar (PESM), SP, e discutimos quais processos podem estar por trás da geração desses padrões em diferentes escalas. No terceiro capítulo, estendemos nossa análise da estrutura genética espacial à escala do interior das moitas e discutimos como os processos ecológicos que determinam a estrutura genética espacial em maiores escalas podem afetar também a organização interna das moitas de bambu. As análises revelaram a existência de uma acentuada estrutura genética espacial em fina escala, em que o grau de parentesco entre indivíduos é significativo até um limite de 11m de distância. Esse padrão pode ser explicado pela ausência de adaptações à dispersão a longa distância em espécies de bambu, um fenômeno que leva à agregação espacial de genótipos aparentados. Também foi observada a existência de duas subpopulações cujo padrão de distribuição possivelmente está ligado à ocorrência de assimetrias na distribuição de condições e recursos ao longo da paisagem. Nossos resultados demonstram que os processos ligados à estruturação genética espacial repercutem igualmente na organização interna das moitas de bambu: em mais da metade dos casos, as moitas analisadas não constituem um indivíduo único, mas contêm uma coleção de genótipos intimamente aparentados. Esse padrão também pode ser compreendido como resultado da limitação de dispersão e

pode estar relacionado a uma estratégia evolutiva mais ampla deste grupo de plantas. Nosso estudo revelou que diversas forças podem atuar simultaneamente determinando a estrutura genética espacial de uma população em diferentes escalas espaciais: enquanto a limitação da dispersão constitui o fenômeno determinante em escalas de alguns centímetros até poucos metros, a heterogeneidade ambiental parece ser o fator governando a distribuição de grupos genéticos mais amplos.

Palavras-chave: Bambus, *Merostachys neesii*, microssatélites, isolamento-por-distância, heterogeneidade espacial, clonalidade.

ABSTRACT

Genetic diversity is one of the three levels of biodiversity and constitutes the driving force behind the emergence and adaptation of species to their habitats. The interaction between the genetic variability present within a population and elements from the environment where it occurs will eventually be translated into a spatial genetic structure; i.e., the non-random distribution of genotypes in space. Information about a population's spatial genetic structure represents a valuable tool to better understand population dynamics and the evolutionary history of a species. The aim of this study was to investigate how the genetic structure of a population is organized across different spatial scales and how these scales relate to each other. We chose as a study model the woody bamboo *Merostachys neesii* (Poaceae: Bambusideae), a species endemic to the Atlantic Forest whose life cycle, reproductive traits, clonal habit and organization in clumps renders an excellent model to test hypothesis about genetic structure in different spatial scales. In the first chapter, we describe the molecular markers used in the genetic analyses of the subsequent chapters. In the second chapter, we analyse the spatial genetic structure of a *M. neesii* population localized in a portion of Montane Atlantic Forest at Serra do Mar State Park – SP, Brazil, and discuss which process may be behind the generation of such patterns in different scales. In the third chapter, we extend our analysis of spatial genetic structure to the interior of the clumps and discuss how ecological process that determine genetic structure in broader scales may also affect the internal organization of bamboo clumps. Our analysis revealed the existence of a strong fine-scale spatial genetic structure, in which kinship levels between individuals are significant up to a 11m distance threshold. This pattern may be explained by the absence of adaptations to seed dispersal in bamboo species, which leads to the spatial aggregation of related genotypes. We also observed the occurrence of two broader subpopulations whose distribution across the plot is most likely related to assymmetries in the distribution of conditions and resources across the landscape. At last, our results demonstrate that the processes related to spatial genetic structuring in broader scales also affect the internal structure of bamboo clumps: in more than half the cases, analysed clumps did not constitute a single individual, but rather a collection of closely related genotypes. This pattern may also be explained as a result of the limited dispersal typical of bamboo species and may be related to a broader evolutionary strategy present in this group of plants. Our study revealed that several forces may act simultaneously to determine the genetic structure of a population in different spatial scales: while limited

dispersal is the main determinant phenomenon in the scale of a few centimeter up to a few meters, environmental heterogeneity seems to be the factor governing the distribution of broader genetic groups in this population.

Key-words: Bamboos, *Merostachys neesii*, microssatelites, isolation-by-distance, spatial heterogeneity, clonality.

SUMARIO

INTRODUÇÃO GERAL

"[...] argumentarei que qualquer pequena migalha de diversidade biológica é inestimável, e deve ser conhecida e acalentada. Não podemos renunciar a ela sem luta."
(Edward O. Wilson, 1992)

A diversidade genética é considerada pela *International Union for Conservation of Nature* (IUCN) como um dos três níveis que compõem a biodiversidade na natureza (MCNEELY et al., 1990). É através da manutenção da variabilidade alélica que as populações são capazes de responder a variações ambientais e persistir em suas comunidades, interagindo com outras espécies e mantendo a estabilidade e a resiliência do ecossistema (HUGHES et al., 2008; SCHABERG et al., 2008). Nesse sentido, deve-se compreender a diversidade genética não como um atributo fixo das populações, mas como um elemento dinâmico que se estrutura no espaço e no tempo em resposta às condições e aos recursos em constante mudança do ambiente (LOVELESS & HAMRICK, 1984). Em organismos sésseis como as plantas, o componente espacial da estruturação genética é particularmente importante, pois estas se encontram completamente dependentes das condições locais do ambiente em que estão inseridas e, não podendo mover-se, estabelecem com este uma interação íntima e permanente (ABREU et al., 2014). À distribuição espacial não aleatória dos genótipos de uma população dá-se o nome de Estrutura Genética Espacial (SGS – do inglês *Spatial Genetic Structure*).

Essa íntima relação entre genoma e ambiente constitui a matéria-prima para os fenômenos evolutivos (EPPERSON , 2003). Por isso, informações sobre a SGS de uma população podem ajudar na compreensão de aspectos-chave da história evolutiva das espécies, tais como fluxo gênico, seleção e deriva (EPPERSON , 2000). Essas análises também são essenciais para se definir estratégias de conservação que visem a manutenção das dinâmicas ecológicas e evolutivas naturais da comunidade (ESCUDERO et al., 2003).

Uma das causas mais comuns da SGS em populações de plantas é a restrição do fluxo gênico causada pela limitação de dispersão dos propágulos (VEKEMANS & HARDY, 2004). Nesses casos, os padrões e a intensidade da SGS dependerão da

interação entre as estratégias de dispersão da espécie e os elementos da paisagem na qual a população está inserida – tais como variações de altitude, padrões microtopográficos ou ocorrência de fragmentação ou outros distúrbios (LOVELESS & HAMRICK, 1984; YOUNG & MERRIAM, 1994; TROUPIN et al., 2006; REIS et al., 2015). Espécies cujos propágulos não alcançam longas distâncias – seja porque o agente dispersor move-se pouco ou porque estão presentes barreiras físicas ao movimento – e que, portanto, permanecem próximos à planta-mãe, acabarão por exibir uma tendência à agregação espacial de genótipos aparentados (HARDY & VEKEMANS, 1999). O processo de deriva genética, através do qual as frequências alélicas variam de forma aleatória ao longo do tempo, terminará por determinar a existência de grupos genéticos distintos, tão diferentes entre si quanto maior for a distância geográfica que os separa (SEXTON et al*.,* 2014).

O padrão de fluxo gênico dentro de uma população também pode ser determinado por assimetrias nas características do ambiente em que a população se encontra inserida (TEMUNOVIĆ et al., 2012). Quando as diferenças ambientais entre porções da comunidade são suficientemente importantes, um processo de adaptação local pode vir a acontecer, levando à formação de grupos genéticos que se organizam de acordo com os padrões de distribuição diferenciada de condições e recursos ao longo da paisagem (ORSINI et al*.,* 2013). Neste cenário, imigrantes e híbridos oriundos de outros ambientes e adaptados a outras condições serão negativamente selecionados e terão grande dificuldade em se estabelecer, o que em última instância representa uma barreira significativa ao fluxo gênico entre subpopulações e reforça o padrão de SGS (SEXTON et al., 2014). Embora essa distribuição diferenciada de genótipos na paisagem possa ocorrer em resposta a fenômenos em larga escala (p.ex., atividade vulcânica) (TSUMURA et al., 2014), especial atenção tem sido dada à importância de fatores microambientais na formação da estrutura genética de populações naturais de plantas, tais como variações locais na profundidade, saturação hídrica e pH do solo, fatores microtopográficos e a existência de padrões irregulares de distribuição de nutrientes à escala de poucos metros ou até centímetros (LECHOWICZ & BELL, 1991; LINHART & GRANT, 1996; HUBER et al*.,* 2004).

Outros fenômenos, como a seleção diferencial ao longo dos estádios ontogenéticos, a ocorrência de reprodução clonal, taxas elevadas de mutação somática ou eventos estocásticos podem deixar suas marcas na SGS de uma população de plantas

(ABREU et al., 2014; HUBER et al., 1999). O importante é que a diversidade genética estrutura-se ao longo de diversas escalas espaço-temporais, e que diferentes processos podem estar na origem dos padrões observados em cada escala (ESCUDERO et al., 2003). Entender de que maneira essas escalas se relacionam e quais os fenômenos mais importantes para a manutenção da diversidade genética em cada uma delas é um passo essencial não só para se desvendar a história evolutiva de uma espécie ou conservá-la, mas para compreender como ela se relaciona com o meio em que está estabelecida e como se conecta com outros processos ecológicos que se ocorrem simultaneamente no seio da comunidade.

Os marcadores

Informações baseadas em medidas genéticas podem ser obtidas de diversas maneiras, desde os métodos baseados na observação sistemática dos fenótipos de diversas gerações, até os métodos baseados em marcadores moleculares (SUNNOCKS, 2000). Marcadores moleculares são segmentos únicos de DNA cujos polimorfismos são representativos da variação presente no genoma de um organismo (AGARWAL et al., 2008). Mutações aleatórias no código genético dos indivíduos – acumuladas ao longo do tempo e submetidas aos efeitos de deriva e seleção – eventualmente desdobram-se em padrões populacionais passíveis de análise. O uso de marcadores moleculares adequados, preferencialmente aliado a modelos de genética de populações, é então capaz de fornecer informações de qualidade sobre a história de vida, evolução e relações entre os organismos (SUNNOCKS, 2000; AVISE, 2004). Sendo assim, é importante escolher o marcador molecular mais adequado para o tipo de pergunta que se pretende responder, levando-se em consideração a escala da análise e os parâmetros a serem medidos (SUNNOCKS, 2000).

Microssatélites (em inglês, *Simple Sequence Repeat*, SSR) são sequências de um a seis nucleotídeos repetidos em tandem, presentes tanto em regiões codificadoras quanto não-codificadoras dos genomas de eucariotos e procariotos (ZANE et al., 2002). São marcadores particularmente úteis devido ao seu alto grau de polimorfismo, codominância e relativa facilidade e baixo custo de desenvolvimento e utilização (MCCOUCH et al., 1997). A cada evento de reprodução sexuada os microssatélites sofrem um rearranjo que torna a nova geração genotipicamente diferente da geração parental de uma maneira que é facilmente identificável. Por isso, são marcadores

importantes em estudos de análise genética em fina escala, como identificação individual e determinação de parentesco (SUNNOCKS, 2000). Ainda, se analisados como genes individuais, os microssatélites prestam-se perfeitamente ao cálculo de frequências gênicas e a análises de distribuição e correlação espacial, o que os torna marcadores eficientes para o cálculo de fluxo gênico, estruturação e diversidade genética, bem como outros parâmetros de genética de populações (SUNNOCKS, 2000).

Bambus: modelo de estudo versátil

Bambus (Poaceae: Bambusoideae) são um grupo de ampla distribuição global, mas cuja ocorrência concentra-se principalmente em florestas tropicais e subtropicais (SODERSTROM & CALDERÓN 1979; MCNEELY, 1995). Em muitas partes do mundo, os bambus lenhosos (Tribo Bambuseae) são parte crucial da economia e da cultura local: seus colmos lignificados permitem a construção de casas e utensílios domésticos; barcos, varas de pescar e instrumentos musicais (MCNEELY, 1999) (Figura 1). Sua importância é também ecológica: florestas de bambu desempenham um papel fundamental na ciclagem de nutrientes em florestas tropicais, e, em nível global, representam um importante sumidouro de carbono – uma propriedade ainda mais relevante diante do atual cenário de aquecimento global (ZHOU et al., 2005; PADGURSCHI et al., *in review*). Além disso, representam importante fonte de nutrientes e abrigo para populações animais como insetos e pequenos mamíferos (LOUTON et al., 1996; HILÁRIO & FERRARI, 2010; CESTARI & BERNARDI, 2011).

Figura 1 Distribuição da tribo Bambuseae (bambus lenhosos). Fonte: http://www.eeob.iastate.edu/research/bamboo/maps.html

Os efeitos da presença de bambus na estrutura da comunidade devem-se a uma série de características de história de vida e ciclo reprodutivo próprios desse grupo de plantas (TABARELLI & MANTOVANI, 1999). Esses mesmos atributos que os fazem ecologicamente relevantes também tornam os bambus um modelo extremamente versátil de estudos de genética populacional. Tipicamente, os bambus alternam longos períodos de propagação vegetativa – que podem durar décadas (JANZEN, 1976)-, com eventos de floração gregária e monocárpica, ou seja, todos os indivíduos florescem ao mesmo tempo e morrem logo após a dispersão das sementes (JUDZIEWICZ et al., 1999).

Durante o período vegetativo, os bambus propagam-se clonalmente através de rizomas subterrâneos que dão origem a inúmeros colmos, os quais permanecem unidos dando origem a densas *moitas* (JUDZIEWICZ et al., 1999) (Figura 2). Moitas e touceiras de plantas clonais, como bambus, tradicionalmente têm sido consideradas como correspondendo a um indivíduo geneticamente único (geneta) formado por módulos (rametas) oriundos do mesmo zigoto e, portanto, idênticos entre si (ERIKSSON, 1993). Entretanto, estudos recentes têm demonstrado que este nem sempre é o caso (FRANKLIN et al., 2008). O fenômeno da multiclonalidade; i.e., a presença de múltiplos genótipos em moitas discretas de plantas clonais, tem sido observado com frequência em estudos moleculares de plantas clonais, incluindo os bambus (KREHER et al., 2000; FRANKLIN et al., 2008, LI et al., 2012). Embora a ocorrência da multiclonalidade esteja relativamente bem documentada, poucos estudos têm se preocupado em explorar os padrões de parentesco entre os rametas e de que modo a estrutura genética intra-moita é afetada pelos processos ecológicos que moldam a SGS em outras escalas espaciais (HÄMMERLI & REUSCH, 2003). Uma das explicações para a existência de moitas multiclonais é o entremeamento de rametas de moitas vizinhas; entretanto, esse fenômeno só foi observado em espécies com rizoma longo (tipo leptomófico) (ISAGI et al., 2004). Em espécies clonais de rizoma curto, que tendem a formar moitas ou touceiras compactas, outros fatores podem influenciar a estrutura interna das moitas: a dispersão limitada e/ou agregada das sementes – p.ex., inflorescências que contendo grande abundância de sementes – pode igualmente dar origem um padrão de moitas multiclonais (LI et al., 2012).

Durante os eventos de reprodução sexuada, os bambus produzem flores pequenas, inconspícuas e adaptadas à polinização por vento (JUDZIEWICZ et al., 1999). Os frutos são pequenas cariopses nutritivas que não apresentam nenhum tipo de adorno que favoreça a dispersão a longas distâncias e, por isso, tendem a cair massivamente sob a planta-mãe (SODERSTROM & CALDERON, 1979; KEELEY & BOND, 1999; PADGURSCHI, 2014). A dispersão limitada das sementes pode ser parte de uma estratégia reprodutiva mais ampla dos bambus, que inclui a disponibilização de nichos adequados à germinação mediante a morte do indivíduo parental (JANZEN, 1976; PADGURSCHI, 2014). A limitação de dispersão também tem repercussões genéticas: diante da ausência de dispersores que as transportam para longe da planta-mãe, a agregação espacial das sementes levará necessariamente à formação de uma forte estrutura genética espacial (HARDY et al., 2006). No caso de plantas clonais, a escala de percepção da SGS vai depender também da escala na qual se concebe o *indivíduo* durante as análises: este pode ser uma moita inteira ou um único colmo (HÄMMERLI & REUSCH, 2003). Nesse sentido, bambus revelam-se modelos particularmente úteis em estudos genéticos porque permitem análises simultâneas em diversas escalas espaciais, que podem ou não ser governadas pelos mesmos processos ecológicos e evolutivos.

Após a floração e a liberação das sementes, os indivíduos reprodutivos morrem, abrindo clareiras na floresta (JUDZIEWICZ et al., 1999). Esse evento pode ter importante repercussão na dinâmica de sucessão da mata ao representar uma janela de oportunidade para o estabelecimento de outras espécies (WIDMER, 1997; TAYLOR et al., 2004; GIORDANO et al., 2009). Para além das repercussões ecológicas que possam ter na comunidade, a combinação dos eventos de floração gregária seguida de mortalidade dos adultos e germinação massiva das sementes também tem outra consequência digna de nota: a ausência de sobreposição de gerações nas populações (STERN et al., 1999).

Posto que as condições ambientais naturalmente variam ao longo do tempo, cada nova coorte que emerge no seio de uma população possui uma estrutura genética que é reflexo do momento em que foi gerada. Em populações onde diferentes gerações coexistem, portanto, parte da variabilidade genética observada entre os indivíduos advém precisamente das respostas às condições ambientais diferenciadas dos diversos momentos em que estes foram gerados (ELLNER & HAIRSTON, 1994). Por outro

lado, a utilização de espécies sem sobreposição de gerações, tais como os bambus, torna desnecessária a inclusão da variação temporal no rol de variáveis passíveis de influenciar a SGS. Em outras palavras, ao utilizar bambus como modelo de estudo, estamos olhando para um registro histórico de como certos processos moldaram a estrutura genética de uma geração em um momento preciso, sem ter de nos preocuparmos com o ruído de fundo representado pela existência de outras gerações – cada uma submetida a uma combinação particular de condições.

Portanto, além da inegável importância ecológica, cultural e econômica que representam para as comunidades naturais e humanas em que ocorrem, os bambus também constituem um importante modelo biológico através dos quais podemos testar hipóteses ecológicas e evolutivas. Sua versatilidade também se reflete na variedade de escalas espaciais em que podem ser analisados. Os resultados desses estudos certamente contribuirão com a construção de um arcabouço de conhecimento a respeito do papel da interação entre a diversidade genética e o ambiente na manutenção da biodiversidade em sistemas florestais tropicais.

A espécie

No Brasil ocorre a maior diversidade de bambus das Américas e uma das maiores do mundo. Aqui registram-se pelo menos 232 espécies, dentre as quais 174 são endêmicas (FILGUEIRAS & GONÇALVES, 2004). A maioria dessas espécies ocorre na região da Mata Atlântica, que é o principal centro de diversidade Neotropical de bambus (JUDZIEWICZ et al., 1999). Em algumas dessas florestas, os bambus chegam a dominar a fisionomia da comunidade, afetando processos de regeneração e sucessão ecológica, bem como influenciando os padrões de diversidade e abundância de árvores (TABARELLI & MANTOVANI 2000; GUILHERME et al., 2004; GRISCOM & ASHTON 2006; LIMA et al., 2012; VINHA et al., 2017).

Merostachys neesii Rupr. (Poaceae: Bambusoideae) é uma espécie de bambu lenhoso Neotropical nativa das florestas atlânticas montanas e submontanas, cuja distribuição estende-se do sul da Bahia ao norte do Paraná (JUDZIEWICZ et al., 1999; FILGUEIRAS & SHIRASUNA, 2009). Seus colmos podem alcançar até 10m de altura e três centímetros de diâmetro (LONGHI-WAGNER et al., 2001). Durante seu período vegetativo, *M. neesii* propaga-se através de rizomas do tipo paquimorfo, ou seja, com

pouco espaço entre os nós dos quais emergem os colmos (JUDZIEWICZ et al., 1999) (Figura 2) dando origem a densas moitas compostas por um número variável de colmos (PADGURSCHI et al., *in prep.*). Devido à sua presença massiva e influência na dinâmica da comunidade florestal – um fenômeno bem registrado para outras espécies de bambu tropical -, *M. neesii* faz-se um candidato perfeito ao desenvolvimento de estudos de diversidade genética dentro de um contexto ecológico, visando tanto a elaboração de estratégias de conservação quanto uma melhor compreensão da história evolutiva desse importante grupo de plantas (OLIVEIRA-FILHO et al., 1994; ROTHER et al., 2009; LIMA et al., 2012).

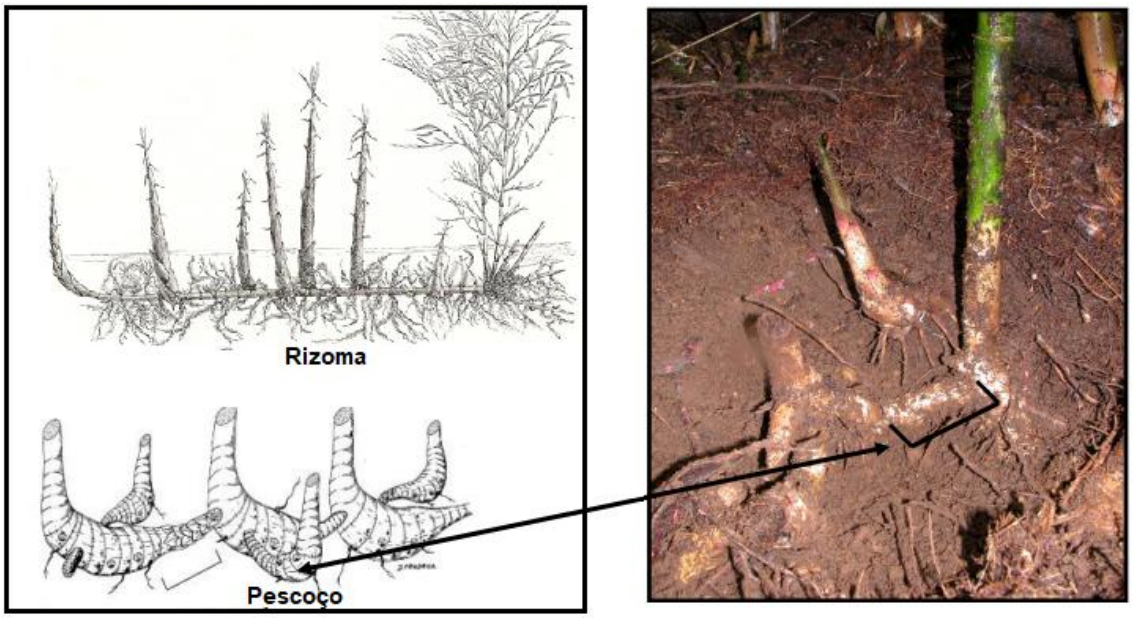

Figura 2 Esquema mostrando a organização geral do crescimento rizomático de bambus e o detalhe do padrão paquimórfico encontrado na espécie estudada. Fonte: Judiziewicz et al., 1999; https://www.bambooaustralia.com.au. Foto: Maíra Padgurschi

A área de estudo

O domínio Atlântico (Mata Atlântica *sensu lato*) constitui a segunda maior formação de vegetação tropical do continente americano e uma das mais ameaçadas, pois seus fragmentos restantes somam pouco mais de 10% da extensão original (RIBEIRO et al., 2009). Apesar dos altos níveis de sobre-exploração e fragmentação, a Mata Atlântica possui um elevado índice de diversidade e um dos maiores níveis de endemismo do mundo (MYERS et al., 2000). Essas características, associadas à alta

produtividade e à grande extensão territorial, tornam a Mata Atlântica um dos mais importantes *hotspots* de biodiversidade do planeta (TABARELI et al*.,* 2005). Dentro desse contexto, e com o intuito de investigar de maneira multidisciplinar os fenômenos geradores e mantenedores da biodiversidade e do equilíbrio ecossistêmico da Mata Atlântica, teve início em 2005 o Projeto Temático Biota Gradiente Funcional (FAPESP 03/12595-7, atualmente parte do Programa PELD/CNPq – sítio FGAF). Através do projeto, foram estabelecidas 18 parcelas permanentes de 1ha cada ao longo de um gradiente altitudinal (0-1100 m) no Parque Estadual da Serra do Mar (núcleos Picinguaba, Santa Virgínia e Cunha), local onde está concentrada a maior parte dos remanescentes de Mata Atlântica do Estado de São Paulo (JOLY et al., 2012). O estabelecimento de parcelas permanentes e da infraestrutura facilitam a execução de estudos sobre a composição e a dinâmica do ecossistema, bem como permite avaliar as mudanças nesses padrões ao longo do tempo (PHILLIPS et al*.,* 1998).

As coletas de material vegetal e as observações ecológicas do presente estudo foram realizadas durante o período de junho de 2016 a outubro de 2017 em uma das parcelas localizadas no núcleo de Santa Virgínia (parcela NSV – 02), que estão inseridas em uma porção de Floresta Ombrófila Densa Montana (Figura 3) (JOLY, 2012). O núcleo Santa Virginia/PESM está majoritariamente (70%) localizado dentro do município de São Luiz do Paraitinga, SP (23°17’ – 23°24’S e 45°03’ – 45°11’W), entre altitudes que variam de 740m a 1600m, e é composto por um mosaico que inclui desde plantações de eucalipto até trechos de floresta primária (JOLY et al*.,* 2012). A parcela utilizada neste estudo está localizada dentro da zona preservada, que no plano de manejo do parque foi declarada *intangível* devido ao alto grau de diversidade (IF, 2018). Por isso, esta área constitui um banco genético a partir do qual se podem realizar estudos visando a conservação e restauração das outras porções da floresta). O clima no local é do tipo subtropical úmido (Cfa ou Cfb segundo o sistema de Köppen), com precipitação média anual de 2300 mm (JOLY et al*.,* 2012).

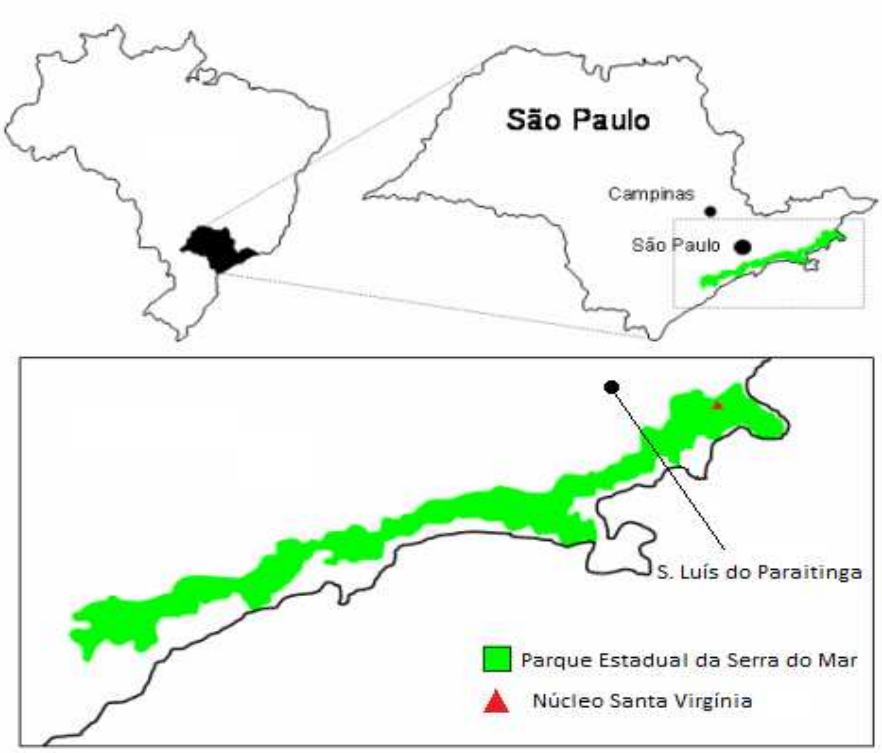

Figura 3 Mapa do Parque Estadual da Serra do Mar em relação ao Estado de São Paulo, mostrando a localização da sede administrativa do Núcleo Santa Virgínia

A mata na área da parcela apresenta fisionomia bem preservada, com baixa intervenção humana, com abundância de bambus, especialmente os da espécie *Merostachys neesii* (PADGURSCHI, 2010). Embora alguns estudos tenham demonstrado efeito negativo da presença dos bambus sobre a riqueza e a diversidade de espécies em florestas tropicais (TABARELLI & MANTOVANI, 2000; GUILHERME et al., 2004; Griscom & Ashton, 2006), *M. neesii* parece não ter relações negativas com o componente arbóreo nas parcelas de Santa Virgínia (PADGURSCHI et al., 2011).

A topografia da parcela é inclinada (>30°) e irregular (EISENLOHR et al., 2013). Essa característica pode estar associada à geração de distúrbios naturais em escala local, especialmente sob a forma de deslizamentos de terra ou quedas de grandes árvores (VIEIRA et al., 2010; EISENLOHR et al., 2013). A presença de distúrbios naturais frequentes, bem como as marcadas variações microtopográficas que caracterizam a área, resultam num padrão de alta heterogeneidade ambiental e a consequente formação de uma variedade de microhabitats (ROCHELLE et al., 2011).

Perguntas, hipóteses e expectativas

Considerando as características do grupo e a sua relevância ecológica nos sistemas florestais em que ocorre, o objetivo do presente trabalho foi analisar a estrutura genética espacial de uma população natural do bambu *Merostachys neesii* em diferentes escalas espaciais através de análises moleculares utilizando marcadores de microssatélites.

No capítulo 1, descrevemos os marcadores desenvolvidos especificamente para a espécie e utilizados no presente trabalho.

No capítulo 2, buscamos responder à seguinte pergunta: como está organizada espacialmente a diversidade genética no seio desta população de *Merostachys neesii* em diferentes escalas espaciais? Além de responder a essa pergunta, levantamos, com base em nossos dados e na literatura especializada, algumas hipóteses acerca dos processos ecológicos que podem estar por trás dos padrões observados. Especificamente, discutimos a possibilidade de que, enquanto a dispersão limitada dos propágulos de *M. neesii* leva à agregação espacial de genótipos aparentados à escala de poucos metros, em escalas maiores a SGS é influenciada por padrões de distribuição não-uniforme dos recursos e condições ao longo da paisagem.

No capítulo 3, o foco é na estrutura genética da população à escala dos colmos e nos debruçamos sobre a possibilidade de que a limitação de dispersão determina o surgimento de moitas *multiclonais* que não são compostas pelos produtos de um único zigoto, mas incluem diversos genótipos aparentados oriundos de sementes-irmãs que brotaram próximas umas às outras. Se este for o caso, esperamos encontrar um número maior de genótipos do que de moitas coletadas, bem como um padrão de elevada similaridade entre os genótipos não-idênticos de uma mesma moita. Por outro lado, se a limitação de dispersão não for uma força preponderante na determinação da estrutura interna das moitas de bambu, esperamos encontrar moitas compostas totalmente por colmos idênticos ou, ainda, esperamos encontrar moitas compostas por colmos que não são significativamente aparentados.

CAPÍTULO 1
CHARACTERIZATION OF NINE MICROSATELLITE LOCI FOR *MEROSTACHYS NEESII* RUPR. (POACEAE: BAMBUSOIDEA)

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ABSTRACT

- *Premise of the study:* Genetic diversity studies are essential to the understanding of how species interact and persist in their environment, contributing to the stability and resilience of the community. *Merostachys neesii* is a Neotropical woody bamboo species native to the Atlantic Forest whose abundance and distinctive life cycle bear great consequence to the structure and dynamics of the community. We thus aim to develop molecular markers that will allow assessment of population genetics parameters as well as eventually serve as tools to futher infer ecological and evolutionary processes.
- *Methods and Results:* We developed a set of nuclear microssatelite markers for *M. neesii*, of which 13 were successfully amplified and 9 were polymorphic. We analised a total of 31 individual genotypes. Number of bands per locus ranged from two to 17. An overall multibanding pattern was observed, with some loci displaying up to six simultaneous bands per individual, strongly suggesting that *M. neesii* is a polyploid species, possibly hexaploid. Polymorphic Information Content values for each locus ranged from 0.37 to 0.89, indicating that these markers are highly informative.
- *Conclusions:* These are among the first microssatelite primers developed for a Neotropical bamboo species, and the first for the *Merostachys* genus. Their highly informative nature makes them useful tools for both conservation genetics and ecological studies.

INTRODUCTION

Genetic diversity is regarded as one of the three levels that make up biodiversity (MCNEELY et al., 1990). It is through the maintenance of allelic variability that populations are capable of responding to envrionmental variations and persist in their communities, interacting with other species and maintaining stability and resilience of the ecossystem. This also means that the study of genetic diversity patterns of a population may offer a generous insight on how a species interact with its surroundings, as well as shed some light on the evolutionary mechanisms behind the surge of certain reproductive traits.

Merostachys neesii Rupr. (Poaceae) is a Neotropical woody bamboo endemic to the Atlantic Forest, mostly occupying montane and submontane altitudes across Bahia, São Paulo and Paraná states (Flora do Brasil 2018). *Merostachys neesii* typically inhabits forests where it plays important roles in many ecosystem processes, providing food and habitat resources for animal species (LOUTON et al., 1996; HILÁRIO & FERRARI 2010; CESTARI & BERNARDI 2011), as well as having a direct impact over forest physiognomies, species composition and overall regeneration patterns (TABARELLI & MANTOVANI, 2000; GRISCOM & ASHTON 2003; LIMA et al., 2012).

This species shows an unusual reproductive cycle, typical of bamboos, with a vegetative growth phase characterized by the production of short subterranean rhizomes, resulting in a dense cluster of culms that, after a long period – about 30 years -, undergoes a synchronous flowering event followed by a massive release of seeds and the subsequent death of all reproductive culms (JUDZIEWICZ et al., 1999; LONGHI-WAGNERet al., 2001). We believe that this reproductive pattern may lead to interesting genetic and ecological repercussions, thus justifying the development of molecular tools to assess levels of genetic diversity and evaluate genetic structure. Furthermore, knowledge of the genetic status of a population is essential for the development of better conservation strategies, especially in the context of a threatened ecosystem such as the Atlantic rainforest (RIBEIRO et al., 2009; PEÑAS et al., 2016).

In this sense, microsatellite markers (SSRs) have proved themselves particularly useful. They are relatively cheap and easy to implement, have higher reproducibility compared to other molecular markers, and because they have a high mutation rate and codominant nature, are considered one of the most informative molecular markers available (VIEIRA et al., 2016). In fact, SSR markers have become the go-to molecular

marker to genotype both cultivated and wild species. In the latter case, the genotypic information thus generated may serve not only to describe basic population parameters, but also to estimate gene flow patterns, kinship coefficients and even evolutionary relations (SUNNOCKS, 2000; VIEIRA et al., 2016).

In this work, we report the development and characterization of nine polymorphic SSR microsatellite loci for *Merostachys neesii*. We hope that these tools lay the groundwork for furthering our understanding not only of this species genetic status and diversity, but also how it interacts with elements of the environment in which it is established and contributes to its balance.

METHODS & RESULTS

We randomly sampled leaves from one *M. neesii* culm from previously established 1-ha plot in Santa Virginia protected area, located within *Serra do Mar* State Park (PESM in Portuguese), São Paulo, Brazil (see JOLY et al., 2012). Genomic DNA was extracted from leaf tissue with BioPur DNA extraction kit (Biometrix Biotecnologia), and was used to develop a microsatellite-enriched library following the Billotte et al., protocol (1999). DNA sample was digested with *AfaI* restriction enzyme and the resulting fragments were ligated to *Rsa*21 adapters. The fragments containing microsatellites were selected by hybridization with (CT)8- and (GT)8-biotinylated probes, followed by capture with Streptavidin MagneSphere Paramagnetic Particles (Promega). The resulting fragments were amplified through Polymerase Chain Reaction (PCR) in 100-µL final volume containing 20µL of selected fragments, 1x PCR buffer, 1.5 mM $MgCl_2$, 200µM dNTPs, 0.4 µmol of primer Rsa21, and 2.5 U of *Taq* DNA polymerase. A C100 Touch thermocycler (BioRad) was used with the following program: 95°C for 1 min for initial denaturation, 25 cycles of denaturation at 94°C for 40 s, primer annealing at 60°C for 1 min; extension at 72°C for 2 min, and a final extension of 72°C for 5 min. The amplicons were cloned into pGEM-T (Promega) vectors. Plasmids were then transformed into *Escherichia coli* XL1-Blue competent cells by electroporation, using a Bio-Rad *E. coli* Pulser (BioRad). The clones were grown in a LB medium containing ampicillin and tetracyclin (both at a 100 mg/L concentration). X-Gal was added to the plate to allow for white-blue screening of clones. Positive clones were selected through their inability to produce functional β-galactosidase and the resultant white color of positive colonies. After DNA extraction

and purification, a total of 192 positive clones were bi-directionally sequenced using an automated ABI 3500xLsequencer (Applied Biosystems).

The sequences were edited to remove vector sequences and low-quality regions using Chromatogram Explorer software (Heracle BioSoft S.R.L., Romania). Cap3software (HUANG & MADAN, 1999) was then used to assemble contigs generated by the bi-directional sequencing. To assure the identity of the samples, the consensus-sequences were then comparatively aligned with sequences from the Genbank database through the BLASTn online tool (ALTSCHUL et al., 1990). Microsatellite sequences were identified with SSRIT – Simple Sequence Repeat Identification Tool (TEMNYKH et al., 2001). Corresponding primers were designed with Primer3Plus tool (UNTERGASSERET al., 2012).

Subsequent DNA samples were extracted using the CTAB method (DOYLE & DOYLE, 1990). Temperature tests were carried out to establish the optimal annealing temperature (*Ta*) for each pair of primers. PCR amplifications were performed in a 9.8µL volume containing 20 ng DNA, 0.75x PCR buffer, 0.1 mM dNTP, 0.2 mM each primer, 0.04% BSA, 1.5 mM $MgCl_2$, and 0.4µL *Taq* DNA polymerase.A. C100 Touch thermocycler (BioRad) was used with the following program: 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 45 s, 1 min at a specific annealing temperature (*Ta*), extension at 72°C for 75 s, followed by a final extension of 72°C for 10 min.

PCR products were first verified by electrophoresis on 3% agarose gels. Of 24 primers, 13 amplified at the expected region, nine yielded no visible bands and two yielded blurred bands that were excluded from the analysis. The 13 amplifying, clear primers were then assessed for polymorphism using the silver-stained 6% denaturing polyacrylamide gel technique proposed by Creste et al., (2001) over 31 culms from distinct clumps. Of these, 9 were polymorphic and 4 were monomorphic (Table 1); i.e, 69% of amplifying primers exhibited polymorphic patterns. All 13 band-yielding microsatellite loci revealed multiband patterns, with each individual presenting up to six simultaneous bands per locus. Lian et al., (2001) remarked that such banding pattern usually implies that the species is polyploid, with each band representing a different allele. For *M. neesii*, this means that the species is probably hexaploid. This finding is consistent with the literature, which suggests that tropical woody bamboo species are mostly hexaploid (SILVA, 2007; YEASMIN et al., 2015). Nevertheless, the exact

ploidy levels of *M. neesii* should be further investigated through more specific cytogenetic methods.

Because of the assumed polyploidy of *M. neesii*, microssatelite markers were treated as multilocus fingerprints and inserted into a binary matrix where each allele was scored present or absent. A total of 83 bands were observed, with the mean number of bands per locus being 9.2 (Table 2); minimum and maximum number of bands per locus were 2 and 17, respectively. Polymorphism Information Content, which measures a marker's efficiency (ranging from zero to one) in terms of how informative they are, ranged from 0.3726 to 0.8976, with a mean value of 0.7275 (±0.18). Markers with a PIC value of over 0.5 are usually considered to be highly informative, while markers with values ranging between 0.25 and 0.5 are considered reasonably informative (BOTSTEIN et al*.,* 1980). Because a polyploid genome presents a significant challenge in determining allelic dosage, we chose not to include heterozygosity calculations in our description of the markers (MCGREGOR et al*.,* 2000). Instead, as proposed by Bussel (1999), we calculated Shannon-Weaver's information index (I) for each locus. This approach enables a comparison among the different levels of diversity detected by each SSR marker. We also calculated gene diversity per locus (H) as proposed by Nei (1973). This index is analogous to the expected heterozygosity and can be interpreted as a mismatch coefficient, i.e., the probability that, for a given marker, two randomly chosen bands are different in the population (KOSMAN, 2003; BONIN et al*.,* 2007). Although there seems to be no direct correlation between the number of alleles and PIC values for SSR markers, PIC, H and I values suggest that, for this population, the more polymorphic markers tend to also be those that convey the more information (PRASAD et al., 2000). Finally, we calculated the number of effective alleles (Ae) per locus, which may serve as an estimate of the prevalence of low-frequency (rare) alleles (PÉTIT et al*.,* 1998). We found that number of effective alleles differed greatly from the number of observed bands, which indicates the presence of several rare alleles in all but one SSR marker.

CONCLUSION

The microsatellite markers described in this work are among the first developed specifically for a Neotropical bamboo species. The multiple banding patterns strongly suggests hexaploidy, which is consistent with findings for other bamboo species. PIC, H

and I values indicate that these primers constitute highly informative molecular markers that may be used in all kinds of population genetics studies, including ecological ones. The marked difference between the number of observed bands per locus and the number of effective alleles suggests the existence of many rare alleles. This reinforces the importance of preserving natural populations and their genetic potential, which may be lost if population is reduced or fragmented.

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Table 1. Description of 13 amplifying microsatellite markers developed for Merostachys neesii over a population of 31 culms (genotypes)

PRIMER	MOTIF	POLYMORPHIC?	SIZE RANGE (BP)	SEQUENCE (5'-3')	SEQUENCE (3'-5')
MN 1	$(CA)_8$	Y	230-250	GTTAGGCCATGGCAATGTTT	CAACCAGATTCTGCCTGTTG
MN2	$(AC)_8$	N	152-162	GCACAGGCCTCAACTAATCT	GACTCTTCCATACCTCAAGTGA
MN3	$(CT)_{13}$	Y	210-228	GGTGGTCATGTGAATCGTTC	CAAGCCAAAGACCAAGGATG
MN9	$(CT)_7$	Y	128-148	GCTATTGCCGAACTAGCCTA	AAAGCCCTCTCAATTCGCTA
MN15	$(GA)_{19}$	Y	240-310	TGCAAATTTCCTCGGACTTT	GTGATTGGAATTATGCAGAGAG
MN16	$(TG)_9$	Y	132-154	ACGAATGGCAAAACAACGAA	GACCACTCACTGCCTACTTT
MN17	$(GA)_9$	Y	268-278	TGACTAATGAAAGCTCGTGGA	TGCTCCCCATGCTATGATTT
MN19	$(GA)_{19}$	Y	160-220	CACAACACACCCAAGAAACA	GAGAAGCTGCACTCGAGTC
MN20	$(TG)_7$	Y	100-150	ACGTGTCAGTGATGTCTCTC	CTAGGCTGTTAGGCAAAGGA
MN21	(GT)5	N	150-170	CTGAGTTCTTGATCTGCTGC	ACCTTCATCTTGATTGCCCA
MN22	$(TC)_{15}$	Y	100-160	TGGCGGATGGACTAATTTCA	AGCCCACCATTGATGTTGTA
MN23	$(TC)_{14}$	N	105-135	CTGCTGCTGTTGTTGCCT	TTGTGTGTGATGGGGAGATC
MN24	(CT)7	N	90-110	GATAGGTTAGCTAGGGTGCC	TGCATCCTTTGTAATGTCATGG

Note: Only markers that yielded clear bands were included in this table

Table 2: Descriptive statistics of nine polymorphic loci, estimated over 31 *M. neesii* culms

Locus	N_{BI}	N_{BL}	PIC	I	H	A_e
MN 1	1-3	4	0.5062	1.03	0.60	2.45
MN3	2-4	6	0.6772	1.55	0.76	5.59
MN9	2-4	9	0.8176	1.94	0.84	6.14
MN15	1-4	14	0.8518	2.28	0.88	7.39
MN16	1-4	4	0.6974	1.38	0.76	3.9
MN17	1-2	2	0.3726	0.69	0.51	1.98
MN19	1-2	13	0.8669	2.30	0.90	8.21
MN20	2-6	14	0.8603	2.42	0.91	7.87
MN22	1-5	17	0.8976	2.65	0.93	10.5
Mean value (SD)		9.2 (5.45)	0.72 (0.18)	1.8 (0.67)	0.78 (0.14)	6 (2.8)

N_{BI}= Number of simultaneous bands for one individual; N_{BL}= Number of bands per locus; PIC = Polymorphic Information Content index; I = Shannon's genetic diversity index; H = Gene diversity index (Nei, 1973); Ae= Number of effective alleles.

CAPÍTULO 2

SPATIAL GENETIC STRUCTURE OF A NEOTROPICAL BAMBOO SPECIES: DIFFERENT PATTERNS AT DIFFERENT SCALES

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Abstract

Complex interactions between life history traits, ecological variables and historical events shape the distribution of genetic diversity and may lead to a non-random spatial distribution of genotypes in a population; i.e., spatial genetic structure (SGS). Understanding how SGS patterns are formed allow us to infer about key ecological and evolutionary processes such as gene flow, inbreeding, and local adaptation. Two main conceptual frameworks have been proposed to explain how genetic diversity is distributed across the landscape: Isolation by Distance (IbD) and Isolation by Environment (IbE). The first describes how limited dispersal creates a pattern of genotypical aggregation in space, with geographic closeness being strictly related to genetic similarity. The second one emphasizes the role of environmental heterogeneity in creating specific niches where processes such as local adaptation may take place. In this work we describe the spatial genetic structure of a native bamboo species at two spatial levels and discuss how the ecological phenomena described by these two conceptual frameworks may explain our results. To assess genetic diversity levels and to evaluate spatial genetic structure, we performed molecular analysis of 33 clumps of *Merostachys neesii*, an endemic and ecologically important bamboo species from the Atlantic Forest. We found high levels of genetic diversity when compared with other long-lived herbaceous perennials and similar to values found for another Neotropical bamboo species (H = 0.268 and S = 3.390). Autocorrelation analysis showed that SGS is strong up to a 11m limit which is consistent with the pattern predicted by the IbD model. Bayesian cluster analysis revealed the existence of two well-defined subpopulations with little gene flow between them, whose range of occurrence cannot be explained by limited dispersal alone. We argue that genotypical distribution at this level is due to asymmetries in distribution of resources and conditions across the landscape. Nevertheless, further studies are required to confirm this hypothesis and to establish which environmental traits are responsible for the pattern found for this bamboo population.

Keywords

Merostachys neesii, bamboos, spatial genetic structures, Atlantic Forest.

Introduction

Genetic diversity has long been recognized as an important factor shaping many characteristics of a population (EPPERSON, 2000). It may impact the productivity, growth, stability, and resilience of a population, as well as influence interspecific interactions within the community and even at the ecosystem level (HUGHES et al., 2008). Often, the complex interaction between life history traits, ecological variables and historical events will shape the spatial distribution of such genetic diversity, leading to a non-random spatial distribution of genotypes; i.e., the population spatial genetic structure (SGS) (RAMIREZ-BARAHONA & EGUIARTE, 2015). Understanding how SGS patterns are formed allow us to infer about key ecological and evolutionary processes such as gene flow, inbreeding, and local adaptation (LOISELLE et al., 1995; EPPERSON 2000). For this reason, SGS studies have become essential to support the development of conservation strategies for endangered and ecologically important species (BALDAUF et al., 2014).

In plant populations, pollen flow and subsequent seed dispersal are the main forces generating genetic diversity and promoting gene flow: it is the movement of propagules that ultimately determine the extent to which genes are locally or more widely dispersed (LOISELLE et al., 1995). In cases where the dispersal of pollen – and, consequently, gene flow – is restricted, one can expect to find a correlation between spatial and genetic distance: the closest individuals in space are also more likely to be related (HARDY & VEKEMANS 1999). This pattern, which is called *Isolation by Distance* (IbD) and assumes the absence of natural selection, arises as the result of local genetic drift determining population differentiation under restricted gene flow, in such a way that geographic distance becomes the major or sole predictor of genetic relatedness between individuals (SEXTON et al., 2014). *Isolation by Distance* (IbD) has been extensively documented in a wide array of species, in both subdivided and continuous populations (HARDY & VEKEMANS 1999; MEIRMANS 2012).

Several life histories and ecological traits are associated with limited dispersion and can thus be expected to predict varying levels of SGS (LOVELESS & HAMRICK 1984). Wind-pollinated species, for instance, have long been considered as harboring low levels of SGS, presumably because they are usually associated with dry, open spaces, where the wind can carry pollen grains over long distances (WHITEHEAD 1969). Seed dispersal mechanisms may also help to shape SGS in some predictable

ways: while a gravity-dispersed species will tend to display a strong SGS because propagules tend to stay close to their parent tree, plants dispersed by birds and large mammals will show weak genetic structure, because the regular long-distance transport of seeds tend to promote homogeneity (LOVELESS& HAMRICK, 1984; HARDY et al., 2006). Other factors related to phenology, breeding system, and floral morphology may also indirectly impact SGS patterns because they influence how and when gametes will encounter and what levels of genetic diversity will be generated (LOVELESS & HAMRICK, 1984).

While IbD remains an important conceptual framework through which we can formulate and test hypothesis, we must not forget that geography is only one of the factors shaping a population's SGS (WANG & BRADBURD, 2014). Landscape features have been shown to influence spatial distribution of genetic diversity in many levels – from small variations in soil composition and resource availability, up to mountain ranges and climatic gradients (TROUPIN et al., 2006; HUBER et al., 2004; REIS et al., 2015). In fact, the subject of local differentiation of genetic subsets of a sympatric population that varies according to environmental differences is not recent (CLAUSEN & HIESEY, 1958; ANTONOVICS 1971). The development of molecular tools and its constant improvements have allowed analysis at finer scales, sometimes smaller than few meters or even centimeters (LINHART & GRANT 1996; BIZOUX & MAHY 2007; MATESANZ et al., 2011). Because plants are sessile organisms, they rely heavily upon small-scale patterns of variations in soil chemistry, nutrient availability and light conditions (HUBER et al., 2004). This idea that environmental variations may shape how genetic diversity is distributed in space; i.e., that genetic differentiation increases with environmental differences, irrespective of geographic distance, has been called *Isolation by Environment* (IbE) (WANG & BRADBURD, 2014).

The link between spatial heterogeneity and genetic diversity is only one of the many *consequences of being a plant*, as described by Bradshaw (1972). Other important consequences that bear importance to this study include their propensity to show considerable chromosomal flexibility, which often results in polyploid patterns, and their flexible breeding systems, that allow them to display varying levels of sexual and asexual reproduction, including the ability to propagate clonally (LINHART & GRANT 1996). The many unfoldings of these traits often have important genetic and ecological repercussions: the polyploidy can be a major source of genetic novelties, and it can act

as a force behind the adaptative prowess of certain plant groups such as *Poaceae* (Levin 1983; LEVY & FELDMAN 2002). In its turn, clonality and clonal architecture is being increasingly considered as an important force driving the evolution of certain reproductive traits such as the delayed flowering of bamboos (TACHIKI et al*.,* 2015).

Given the diversity and complexity of the processes involved in structuring genetic diversity across a population, it is often advised to researchers that one must carefully chose in which spatial scale to conduct analysis. Different ecological processes may be acting simultaneously at different spatial and temporal scales, and may even influence one another (WIDEN et al*.,* 1994; ESCUDERO et al*.,* 2003). In this sense, clonal species may be of special relevance when testing hypothesis about SGS because their very existence takes place in two scales: the genetic entity (genet), that may sometimes spread across thousands of kilometers, and the vegetative modules of the genetic individual (ramets) (HÄMMERLI & REUSCH 2003).

Merostachys neesii Rupr. (Poaceae) is a Neotropical bamboo species native to montane and submontane tropical Atlantic forests (JUDZIEWICZ et al*.,* 1999; Filgueiras & Shirasuna 2009). As it is typical of many bamboo species, *M. neesii* has a life cycle that is characterized by long vegetative periods – of around 30 years – punctuated by monocarpic gregarious sexual events (JUDZIEWICZ et al*.,* 1999; LONGHI-WAGNERet al*.,* 2001). During the vegetative phase, *M. neesii* grows clonally through short, clump-forming underground rhizomes; during the flowering events, it produces typical wind-pollinated inconspicuous flowers with an abundance of dust-like pollen (JUDZIEWICZ et al*.,* 1999; PADGURSCHI et al*., unpublished data.*). Seeds lack adaptations to biotic dispersal and tend to fall massively under the parent clump (Liebsch & Reginato 2009; PADGURSCHI 2014). After seed setting, reproductive individuals die, opening gaps whose colonisation is a major promoter of diversity in many communities (WIDMER 1997; GUILHERME et al*.,* 2004). This distinctive series of events – mass flowering and seed setting followed by the death of the reproductive individuals – also means that there is almost to none generational overlapping in this species (STERN et al*.,* 1999). This is a specially interesting property because whatever SGS is observable in this population will be the reflex of a specific set of ecological conditions found during the seedling recruitment phase, instead of being the sum of several overlapping SGS reflecting the processes that took place during the recruitment of each cohort (ELLNER & HAIRSTON, 1994).

Notwithstanding their important role in the community dynamics and their suitability as genetic models, there is a shortage of information on the reproductive biology of bamboos, mainly because sexual events are just so rare (JANZEN 1976). Likewise, there is little available genetic data for this group, and most genetic studies on tropical grasses focus on a few crop species that have been thoroughly selected by men (GLAZMANn et al.,1997; GLÉMIN & BATAILLON, 2009). The aim of this study is to assess genetic diversity of a natural population of an ecologically important bamboo species, as well as investigate how different ecological processes interact to give rise to patterns of SGS at different scales. Given the species limited dispersal, we expect to find a pattern of Isolation by Distance at small spatial scales. We also expect to find some level of local genetic differentiation at a broader scale, related to the high levels of heterogeneity typically found in tropical forests. Finally, we expect to find some degree of polyploidy, which is a pattern commonly reported for bamboos and most grass species.

Material and Methods

Study site – The study was conducted within 1-ha permanent plot (plot M; see JOLY et al., 2012), in a portion of Montane Atlantic Forest at the *Serra do Mar* state park, municipality of São Luís do Paraitinga, São Paulo state (23º 13' 18" S, 45º 18' 36" W). Altitudes within the plot range from 990 to 1093m (JOLY et al., 2012). The area is covered by dense ombrophilous vegetation dominated by bamboo clumps (PADGURSCHI et al., 2011). The climate corresponds to Köppen's Cfb type, mean rainfall exceeds 2200mm per year and soil is classified as a dystrophic haplic cambisol (SALEMI et al., 2013; MARTINS et al., 2015). Topography is very steep (> 30°), marked by numerous slopes and valleys, including scars of landslides (EISENLOHR et al., 2013). Although there have been reports of selective logging during the early XX century, the area displays a physiognomy typical of well preserved forests (PADGURSCHI et al., 2011).

Study species – *Merostachys neesii* R. (Poaceae:Bambusoideae) is an endemic bamboo species of the Bambuseae tribe that occupies montane and submontane Atlantic forests across a range that goes from southern Bahia state to northern Paraná (Flora do Brasil 2018). It occurs naturally in Neotropical forests, where it may sometimes dominate the

landscape and affect density, diversity and local richness of pioneer species (TABARELLI & MANTOVANI 1999; OLIVEIRA-FILHO et al., 1994). *M. neesii* grows clonally through short underground rhizomes of the pachymorph type, with short internodes through which culms emerge and give rise to tightly packed clumps (PADGURSCHI 2014). Culms can reach up to 10m of length and 3cm of diameter (LONGHI-WAGNERet al., 2001). After about 30 years of vegetative growth, a massive sexual event occurs, and culms produce abundant, albeit inconspicuous, flowers of the spiklet type. These flowers, as do typically wind-pollinated species, produce copious amounts of fine, light weight pollen (JUDZIEWICZ et al., 1999). Seeds are small, gravity-dispersed caryopsis that are sometimes eaten by rodents (CESTARI & BERNARDI, 2011; Shirasuna & Filgueiras, 2013). After seed setting, all reproductive culms die, an event that has been described as 'large-scale disturbances' and bears great importance to forest dynamics (GONZÁLEZ et al., 2002).

Sampling – We conducted an asystematic sampling of 31 clumps across the whole plot. We considered that culms that disted less than 0.5m from one another were part of the same genet (clump) (PADGURSCHI et al., in prep.), and that culms apart more than 1m from another one as different individuals. Ortogonal coordinates were taken for each clump. We collected leaf tissue samples from one random culm within each clump. All samples were brought to the laboratory within 12-hours after collected and kept in a bio-freezer at -80°C until DNA extraction.

Molecular analysis – DNA samples were extracted using the CTAB method (DOYLE & DOYLE 1990). Genetic variation and structure were assessed using nine polymorphic microssatelite markers developed specifically for *M. neesii* following the Billote et al., (1999) protocol. Primer characterization and detailed procedures can be seen in Borges et al., (Chapter 1). Samples were previously analyzed through agarose gel electrophoresis to confirm SSR amplification and then genotyped using the silver-stained 6% denaturing polyacrylamide gel technique proposed by Creste et al., (2001), using a 10 pb ladder to allow for accurate determination of band size.

Statistical analysis – Because of the challenges involved in determining exact allelic dosages in polyploid species such as *M. neesii*, microssatelite markers were treated as multilocus fingerprints and inserted into a binary matrix where each allele -hence

referred to as markers – was scored present or absent. For this same reason, we did not test for Hardy-Weinberg equilibrium. We chose to calculate the following basic genetic diversity indexes: number of multilocus genotypes, Nei's gene diversity (H), Shannon-Wiener diversity index (S) and Simpson's diversity index (D). While D simply represents the probability that two randomly chosen bands are identical, S also takes into account the evenness of genetic variation within the population; that is, if a single genotype dominates most of the samples or if genotypes are evenly distributed. Diversity indexes were calculated using the poppr statistical package inside the R environment (KAMVAR et al., 2014).

In order to verify the existence of genetic differentiation patterns that might be related to spatial discontinuities in resource distribution, a Bayesian cluster analysis was performed using the STRUCTURE software (PRITCHARD et al., 2000) based on haploid (presence/absence of bands) data. Ten independent runs were performed for each of six values of K (number of clusters), with 250000 burn-in periods and 500000 Markov Chain Monte Carlo iterations. We then used the program Structure Harvester (EARL & VONHOLDT 2012) to determine the most reasonable K, based on ΔK values.

To further explore how genetic variation of the population is distributed, we calculated genetic distances for each pair of individuals as suggested by Bruvo et al., (2004) – an approach that takes into account mutation events and is better suited to polyploid species -, and used the resulting data matrix to run a Principal Component Analysis (PCA). To estimate the relationship between genetic and geographic distances, we performed a Mantel test whose significance was assessed through 19999 permutations of rows and columns, also based on Bruvo's distance. Genetic distance calculations, Mantel test and PCA were performed using the Polysat R package (CLARK & JASIENIUk 2011). Gst value, calculated based on the results of both Bayesian and PCA analysis, was also determined using Polysat package.

Then, in order to investigate fine-scale genetic structure and test for Isolation by Distance, we used the software SPAGEDI to draw an autocorrelogram between kinship values and geographic distance for all pairs of individuals (LOISELLE et al., 1995; HARDY & VEKEMANS 2002). In order to refine our analysis to the smallest scale possible, we established 54 distance classes whose upper limits ranged from 3m to 107m. These limits were chosen in order to create homogeneous distance classes; that is, classes that contain the similar number of pairs each.

Results

Of the 13 amplified primers that yielded clear bands, nine were polymorphic, which means a 69% rate of polymorphism. A total of 83 bands were observed, with an average of 9.2 bands per locus. A persistent pattern of multi-banding was detected in all loci, with up to six bands per sample in some cases, which suggests a highly polyploid species. Upon analysis with all nine polymorphic markers, we were able to detect 30 multilocus genotypes at the clump level, which is a slight departure from the number of total sampled clumps. Nei's gene diversity (H) for these samples was 0.268, while Shannon-Wiener diversity index (S) reached 3.390 and the complement of Simpson's diversity index (D) totaled 0.966.

Autocorrelation analysis between kinship coefficients and geographic distance revealed a strong fine-scale genetic structure in this population, with maximum kinship values occurring within the first distance class (1.0m – 3.2m). From then on, this tendency towards genetic structure plummets steeply and ceases to exist in a significant manner around 12m of distance (Figure 1). Kinship values were not significantly different from zero beyond these distance limits, except for a slight positive correlation at 15.5m and negative correlations at 80.9 and 92.2m.

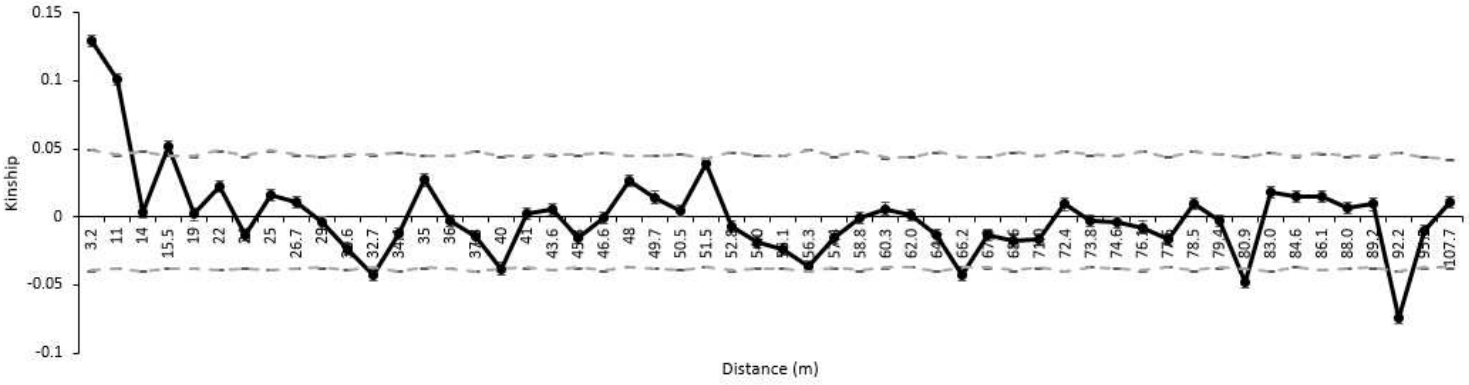

Figure 1 Autocorrelogram showing the relationship between Kinship values and pairwise distance class

Bayesian likelihood analysis of population structure at the genet level supported the existence of two well-defined subpopulations with little gene flow between them (Figure 2). This was supported by the fact that the highest ΔK value was found when K = 2. The biggest subset, hence referred to as *green population*, included 23 individual multilocus genotypes and spreads throughout most of the plot.

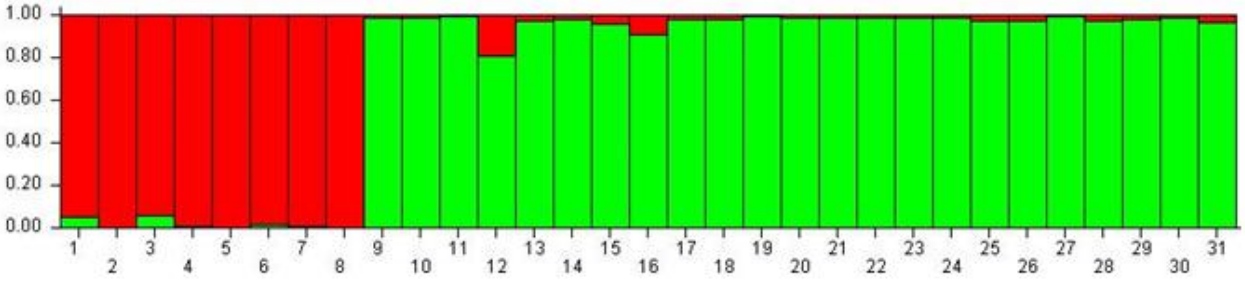

Figure 2 Estimated population structure among 31 genets within the plot, showing two clear genetic subsets (green and red) with little admixture between them.

The smaller subset, hence referred to as *red population*, includes 8 multilocus genotypes and concentrates in the lowest altitude parts of the plot. PCA analysis returned very similar results (Figure 3), with red and green subpopulations including seven and 23 individuals, respectively. Two of the clumps shared the exact same genotype and were thus considered as one individual during PCA analysis, hence the slight difference between population sizes. A global Gst value of 0.039, albeit relatively low, further supported the existence of two separate subpopulations.

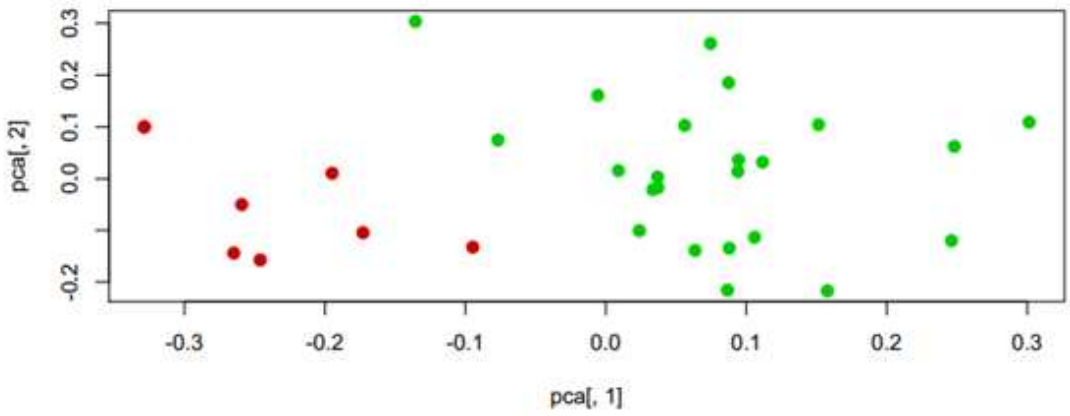

Figure 3 Principal Component Analysis (PCA) for 31 multilocus individuals, showing genetically distinct clusters (green and red points)

Each subpopulation seems to occupy a specific area of the plot, without co-existence between clumps of different groups (Figure 4). Mantel test returned a highly significant ($p < 0.01$) value (0.275) after 20,000 permutations, which further suggests the existence of a correlation between genetic relatedness and geographic distance. Subpopulations also differed in relation to diversity indexes. Red population returned lower diversity indexes (S = 1.91 and H = 0.736) than green population (S = 3.14 and H = 0.787). Proportion of rare alleles was higher for green population: 21% against 10%

for red population. However, caution should be exercised when interpreting H values for small samples of polyploid individuals. It is also not clear how the differences in the number of individuals of each group influenced these contrasting results.

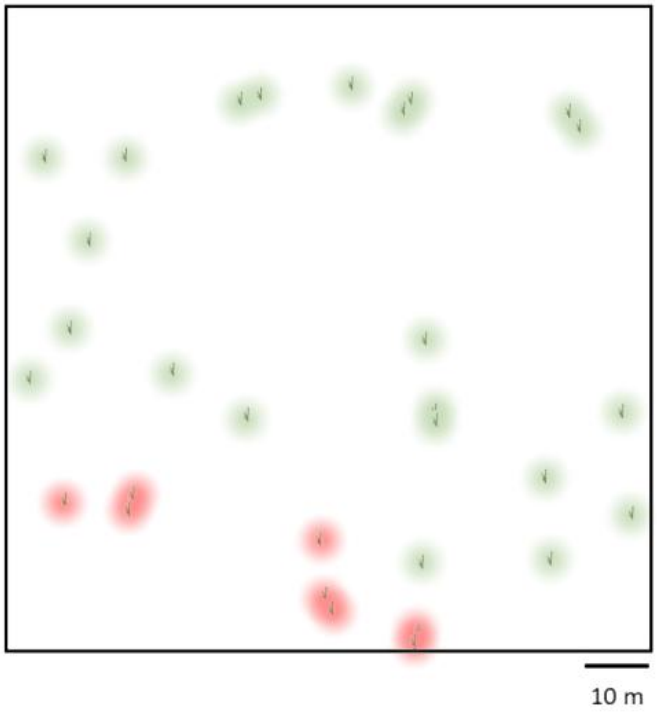

Figure 4 Map of the plot showing the distribution of sampled clumps within it. Green and red colors represent their respective genetic subset as determined by both Bayesian and Principal Component analysis.

Discussion

Polyploid patterns are common in grass species, with some authors proposing that virtually all *Poaceae* members are polyploid to some extent (LEVY & FELDMAN 2002). This is also the case for bamboo species, with literature suggesting that ploidy level varies along a latitudinal gradient: while temperate bamboos tend to be tetraploid, tropical species tend to be hexaploid (YEASMIN et al., 2015). This pattern was observed in 32 out of 36 tropical species from the Bambuseae tribe that had their ploidy levels assessed through cytogenetic techniques (da SILVA 2007). Results from these studies, coupled with the multibanding patterns consistently observed for several individuals of *M. neesii* during the genotyping process, suggests that this is a hexaploid species. Nonetheless, exact ploidy levels for this species have yet to be exactly determined through appropriate cytogenetic methods.

The high ploidy level of *M. neesii* has both ecological and methodological consequences: polyploidy is often associated with invasive and overall early successional species, which may explain the success of *M. neesii* in dominating the physiognomy of this part of the forest (STEBBINS 1985; OLIVEIRA-FILHO et al., 1994). It also means that we must pay close attention to the ploidy of the species when analyzing genetic data because traditional diversity indexes were mainly developed for diploid models and may not be applicable to polyploids (OBBARD et al., 2006).

Few studies assessing genetic diversity of bamboo species have been published to date, making it difficult to establish a pattern against which to compare the data obtained for *M. neesii*. Nei's gene diversity (H) for the whole *M. neesii* population, 0.268, is similar to the genetic diversity value found for another Neotropical bamboo species (H_e = 0.273) (ABREU et al., 2014). The value of the complement of Simpson's diversity index for *Merostachys neesii* (D= 0.966) is also higher then the average value for several self-compatible clonal species (D=0.86; HONNAY & JACQUEMYN 2008). Although to date there has been no study comparing genetic diversity levels of species with overlapping versus nonoverlapping generations, the role of overlapping generations in maintaining genetic variation in fluctuating environments is well established (ELLNER & HAIRSTON, 1994). The fact that a single cohort may harbour as much – if not more – genetic diversity than populations comprised of several overlapping generations, suggests that Neotropical bamboo species have some combination of ecological traits that maximize their levels of genetic diversity.

High genetic diversity levels have been reported for several clonal species, sometimes as high as strictly sexual ones, especially in cases where there is alternation between vegetative growth and sexual events (WIDEN et al., 1994). Mixed-mating woody perennial species have also been shown to harbor more genetic diversity than their non-woody counterparts (Hamrick et al., 1994; BROADHURST et al., 2016). Furthermore, in clonal plants, somatic mutations may arise in some individuals and spread throughout the population by means of assexual propagation (WHITHAM & SLOBODCHIKOFF, 1981; KING & SCHAAL, 1990). However, while very important in promoting genetic diversity in long-lived and/or manly assexual species, the extent to which somatic mutations may impact the genetic diversity of *M. neesii* is not clear (KING & SCHAAL 1990; GROSS et al., 2012). Finally, hybridisation events may also contribute to a greater genetic diversity by combining different genomes and generating novel genetic combinations (VALLEJO-MARÍN & LYE, 2013). In a recent study,

Triplett & Clark (2010) presented numerous evidence that hybridization among bamboo genera is not only possible, but that it constitutes a major force driving evolution and speciation across the temperate clade. The same has been found for tropical bamboo species, albeit mainly paleotropical (THAKUR ET AL., 2016) In fact, hybridisation events have been proposed as the main mechanism behind the much prevalent polyploidy observed among grass species (STEBBINS, 1956). All these factors may explain the prevalence of high levels of genetic diversity in *Merostachys neesii,* as well as other bamboo and clonal grass species (GUTIERREZ-OZUNA et al., 2009; ABREU et al., 2014).

High levels of genetic diversity are commonly associated with improved adaptability to new environments and an overall increment in a species' fitness (REED & FRANKAN 2003). It is also a common measure of a population's ability to persist in the community, specially under stressfull or changing environments (LAVERGNE & MOLOFSKY, 2007; HUGHES et al., 2008). Our findings regarding the genetic diversity levels of this population of *M. neesii* may thus partially explain the abundance of this species in such an heterogeneous patch of tropical forest. Other ecological processes may also be impacted by the high level of genetic diversity observed for *M. neesii*: Crutsinger et al.,(2008) and Hughes & Stachovicz (2004) demonstrated that genetic diversity of dominant species enhances the resistance of the ecossystem to disturbance and establishment of invasive species, and genotypic richness of a plant species have been showed to positively affect species richness levels of herbivore species such as arthropods (CRUTSINGER, 2006). Further studies are nevertheless needed to direclty test the effects of the genetic diversity of *M. neesii* on the community.

The strong pattern of fine-scale genetic structure is probably a result of restricted seed dispersion and pollen flow such as predicted by the Isolation by Distance model (LOISELLE, 1995). Although, as mentioned above, wind-pollinated species tend to exhibit lower levels of SGS, this may not be the case when they occur inside rainforests: its dense structure forming a tapestry of leaves and trunks serves as a barrier to pollen flow, and the intense, year-round humidity renders pollen grains damp and heavy, making it difficult for them to be carried away by the wind (WHITEHEAD, 1969; DICK, 2008). Restricted pollen flow, combined with gravity-dispersed seeds, will naturally result in neighboring clumps being genetically closer than clumps far apart, as is clearly shown by the autocorrelogram.

The existence of two distinct, spatially-segregated subpopulations within a 1-ha plot naturally presupposes the existence of an obstacle that prevents gene flow between members of different groups, or at least an important enough environmental factor that would restrict certain genotypes to specific sites (ORSINI et al., 2013). While geographically close clumps of *M. neesii* are more likely to belong to the same group, restricted dispersal alone cannot explain the existence of two well defined subpopulations. It also cannot explain what confines each cluster to their range of occurrence beyond the 11m limit of significant kinship.

In tropical forests, microtopographic variation seems to be an important source of environmental heterogeneity, as it is generally associated with differences in soil depth and composition, water content, drainage and light availability (CAMPOS et al., 2011). In another study also conducted with *M. neesii*, Padgurschi (2014) noticed that pronounced irregularities in the terrain caused a decrease in luminosity in the lower parts of the plot, where density of individuals is also smaller and where our red population is mostly contained. The soil in this lower part of the plot also displayed lower effective porosity, which may translate into higher water saturation – a factor that may negatively impact the establishment of bamboo clumps (KIEHL, 1979; FRANKLIN & BOWMAN, 2003; PADGURSCHI, 2014). Differences in both light conditions and water availability have been shown to represent a strong enough force to drive significant population differentiation at genetic levels (JACQUEMYN et al., 2005). Furthermore, in a 2012 study, Lima et al. noted that *Gadua tagora*, a native bamboo species, occurs more frequently in clayey soils, probably due to preferential establishment of clumps in these areas.

Whichever environmental variations may exist between different sites of the plot, they may be enough to represent a significant selective pressure, preventing the establishment of immigrants of a certain genotype into a site that is dominated by another set of genes, reinforcing a tendency towards environment-based genetic structuration (ORSINI et al., 2013). This pattern of spatial differentiation in microscale has been extensively documented for several plant species; one of the most elegant and detailed of such studies involved plants that occupy extreme soil conditions such as those contaminated with heavy metals (ANTONOVICS, 1971). In his classical paper, the author demonstrated that under this harsh scenario, a continuous population will be structured in such a way that individuals that are tolerant to a certain contaminant will only occupy sites that have high levels of this particular contaminant, not being able to

establish themselves in sites that are contaminated with other pollutants. This is because tolerance to a specific condition often comes at the expense of changes in physiological pathways that will, in turn, prevent their establishment in other conditions and even hinder their competitiveness in otherwise favorable environments (WANG & BRADBURD, 2014). The same evolutionary mechanisms, albeit in a less extreme fashion, may be at play in this population of *M. neesii*: different tolerance thresholds to certain conditions, such as light availability and soil composition, driving the formation of environment-based genetic clusters (YOUNG, 1991; ROCHELLE et al., 2011; PADGURSCHI, 2014).

Conversely, genotypes may be following a distribution based on a trade-off between resource availability and negative biotic interactions. Naturally, clumps will tend to establish more frequently within favourable sites, which means that these will also be the sites with the highest densities of individuals. High density of individuals often bring on a set of density-dependent negative effects, such as intraspecific competition and increased presence of predators and pathogens (CLARK & CLARK, 1984). Less competitive genotypes may thus be driven towards the fringes of what could be considered a favorable environment, avoiding competitive exclusion but establishing smaller, more spatially restricted populations. The effects of intraspecific competition on SGS have rarely been studied in natural, continuous populations, but Matesanz et al*.,* (2011) noticed that the abundance of a close congener influenced the spatial genetic structure of another plant species, and Bazzar et al*.,* (1982) registered differences in intraspecific genotypic survivorship depending on environmental factors.

Finally, it is of utmost importance to consider the matter of scale itself – both spatial and temporal. When choosing the scale at which to conduct sampling and subsequent spatial and environmental analysis, one must make a compromise between praticality and meaningfulness of data (LEVIN, 1992). Because our study was conducted in 1-ha plot, it is possible that what we detected is actually a fragment of a much bigger picture and a reflex of much broader environmental patterns. Thus, if one wishes to investigate the ultimate causes of such genetic structure, we strongly suggest that samples and field data are collected from a wider range. The matter of time scale may also poses some challenges in interpreting spatial genetic structure patterns. In the case of long-lived perennials with infrequent sexual events and long vegetative periods such as *M. neesii*, the genetic patterns we observe today is a response to environmental conditions at the time of seedling recruitment, as well as the various ecological filters

acting during the first years leading to plant establishment (PADGURSCHI, 2014). These conditions may not be the same at the time of the sampling, making it difficult to distinguish the actual forces acting in the genetic structure, as well as preventing us from realizing the occurrence of any historical events or disturbances that may have taken place during the initial stages of seedling recruitment and been a fundamental force in causing the separation of the two clusters.

Our results thus suggest that, for this population, several ecological processes act simoultaneously at different spatial and time scales. While genetic structure at the scale of a few meters seems to be mostly a result of limited dispersal of propagules, such as described in the classic model of Isolation-by-Distance, the exact forces behind the formation of bigger genetic clusters are not completely clear. We nonetheless propose that this broader geographic distribution of genotypes is related to differences in resource availability and plant density, which may be generating a pattern of environment-based genotypic distribution as described by the Isolation-by-Environment model. Further studies are necessary to confirm this hypothesis and determine which environmental variables or combination of variables are preventing gene flow between subpopulations, as well as to verify the extent to which spatial and temporal scales affect our perception of these genetic patterns.

The results generated in the present study represent an advancement in the knowledge of genetic status of Neotropical bamboo species and contribute to further our understanding of how they interact with their environment. This may help in developping conservation strategies that aim to preserve not only one species in particular, but to maintain the ecological interactions that promote the overall balance of the community.

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CAPÍTULO 3

FINE-SCALE GENETIC STRUCTURE OF *MEROSTACHYS NEESII* CLUMPS

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Abstract

Clonal organisms may be perceived in two levels: the genet, composed of all tissues originating from one zygote, and their modular shoots, or ramets. This organization is at the core of what makes clonality an ecologically important life history trait; on the other hand, it also means that individuality is an ambiguous concept among these species. This fact should be considered when studying populations of clonal plants because what may be perceived as one macroscopic individual may include several separate genotypes. Here we use microssatelite markers to examine the inter-ramet genetic structure of a native bamboo, *Merostachys neesii*. Our aim was to investigate the prevalence of multiclonality among clumps of this Neotropical species and discuss the most probable mechanisms through which this phenomenon may arise. We collected two to five culms from each of 17 clumps from a portion of Montane Atlantic Forest in Southeastern Brazil. Each individual culm was genotyped and inserted into a binary matrix, after which we performed an UPGMA analysis of Jaccard's distance to examine the genetic relationship between the ramets. The results revealed that while over 70% of the clumps were multiclonal, non-identical ramets belonging to the same clump were genetically much closer among themselves than their neighbors were. We propose that the most likely mechanism that explains this pattern is the limited dispersal of propagules observed in this species, which results in siblings establishing very close to each other. This process may be related to a broader evolutionary strategy that has been proposed to explain the life cycle of bamboos.

Key-words: Woody bamboos, inter-ramet, clonality

Introduction

Clonality is a life history trait associated with sessile organisms from a wide array of taxa and habitats, and is defined as the capacity to produce vegetative offspring from a single ancestor (BECHELER et al., 2014; DONG et al., 2014). Species may be asexual, relying only on vegetative growth to establish populations, or they may possess a mixed system that combines, at varying levels, both vegetative growth and sexual recombination (BECHELER et al., 2014). In both cases, clonal individuals are organized in two levels: the genetic individual, or genet, composed of all tissues originating from one zygote, and the modular shoots, or ramets, that are more or less independent parts of the genet (ERIKSSON, 1993). Because clonal individuals may be recognized at these two levels, the very concept of individuality itself is a complex, ambiguous one when dealing with such species (SANTELICES, 1999).

The ecological benefits of clonal growth are related precisely to these species' ability to create what has been called *cooperative extensions* of their individuality (FRANKLIN, 2008). Through ramet extension, clonal plants can better harvest patchily distributed resources and share the mortality risks to the genet among several ramets, and even buffer themselves from spatial and temporal variations in resource availability – abilities that are especially useful in highly heterogeneous habitats (CHESSON & PETERSON, 2002). Therefore, clonal populations may benefit from the improved fitness brought by this life strategy to succeed in highly heterogeneous and/or disturbed environments (OLIVEIRA-FILHO et al.,1994), sometimes up to the point of becoming ecological threats (GUTIERREZ-OZUNA et al., 2009).

Merostachys neesii Rupr. (Poaceae: Bambusoideae) is a Neotropical bamboo species native to montane and submontane tropical Atlantic forests, where it forms large clumps that may influence species richness and diversity in those communities (TABARELLI & MANTOVANI 2000). Its life cycle is characterized by long vegetative periods punctuated by massive gregarious flowering events (JUDZIEWICZ et al., 1999; PADGURSCHI, 2014). During its vegetative phase, *M. neesii* propagates through short underground rhizomes that give rise to dense, tightly aggregated clumps of culms that remain physiologically connected, as in a typical *phalanx* strategy (FISCHER et al., 2004). The actual physical limits of each genet, however, are not so straightforward: because rhizomes are buried underground, one has to rely on eyesight to infer where a clump ends and the next one starts; i.e., they must rely on a

macroscopic perception of individuality (SANTELICES, 1999). This approach, while intuitive, may not reflect the reality that clumps perceived as one genet may sometimes actually be a collection of several different genotypes (a *multiclonal clump).*

Franklin (2008) observed this phenomenon in a study conducted with *Bambusa arnhemica*, a tropical bamboo species, whose clumps were found to include up to five different genotypes – thus contradicting the idea that one clump equals one genotype. LI et al., (2012) found the same result for another grass species, and this pattern of multiclonality have been observed repeatedly for a multitude of clonal plants (WAYCOTT 1995; DEMCHIK et al., 2016). The possibility of multiclonality is especially important in studies looking into genetic diversity levels at small scales: if clumps that contain several genotypes are sampled as one genetic individual, a great deal of information is being left out of the analysis, thus artificially altering diversity indexes. Analysing within-clump structure may also help to shed light on the biological and ecological function of the clumping habit and how it relates to other reproductive traits (WHITHAM & SLOBODCHIKOFF, 1981; FRANKLIN, 2008).

In this work, we use microsatellite markers to examine the genetic structure of *M. neesii* clumps to determine if their macrostructure resonates with their genetic identity; i.e., if what is perceived as a clump is one genetic individual or a composite of genotypes. We also explore the genetic relation among genets and how this relates to the mechanisms through which multiclonal clumps may arise in a population. Our results may help future ecological and genetic studies in clonal plants by further clarifying the limits between each level of clonal identity, allowing researchers to make an informed choice on which type of analysis they want to pursue. Finally, we hope to improve the understanding about the evolution of some of the traits that make the life cycle of bamboos such a peculiar matter.

Material and methods

Study species

Merostachys neesii Rupr. (Poaceae: Bambusoideae) is a Neotropical bamboo species whose range of occurrence spans from southern Bahia to Paraná state, occupying mostly montane and submontane altitudes. After around 30 years of vegetative propagation through short (<50cm) underground rhizomes of the

pachymorph type, massive synchronous flowering ensues (PADGURSCHI - not published data). Flowers are typical grass spikelets without adaptations to biotic pollination and produce an abundance of fine, dust-like pollen. Seeds are small, nutritious caryopsis that falls massively under the parent tree, seemingly lacking dispersal agents (JUDZIEWICZ et al., 1999; PADGURSCHI 2014). After seed setting, parent plants quickly die, causing the opening of gaps in the canopy (JANZEN 1976; JUDZIEWICZ et al., 1999), an event that has been shown to bear great importance to the forest structure and diversity (OLIVEIRA-FILHO et al., 1994).

Study area

The present study was conducted within 1-ha permanent plot (JOLY et al., 2012) in a portion of Montane Atlantic Forest at the *Serra do Mar* state park (PESM, in Portuguese), São Paulo state, Brazil. The plot is covered by dense ombrophilous vegetation, mostly dominated by bamboo clumps of varying sizes (PADGURSCHI et al., 2011; JOLY et al., 2012). Topography is very steep and irregular, traits that are directly linked to the high rates of disturbance and environmental heterogeneity that characterize this part of the forest (EISENLOHR et al., 2013). The climate is of highland subtropical variety (Cfb type), and annual rainfall exceeds 2200mm per year.

Sampling

Following Padgurschi (2014), we assumed that the neighboring culms that were less than 50cm apart belonged to the same clump. To avoid re-sampling of genets, we established a minimal distance limit of 1m between sampled clumps, which were random selected within the plot. Leaf tissue samples were collected from two to five individual culms from each of 17 clumps. Orthogonal coordinates were taken from each discrete clump; culms from a same clump were considered as having the same coordinates. Each ramet received an ID number, which was used to identify the samples during analysis.

Molecular & statistical analysis

DNA samples were extracted using the CTAB method (DOYLE & DOYLE, 1990). Genetic variation among ramets was assessed using nine polymorphic microsatellite markers developed specifically for *M. neesii* following the Billote et al.,

(1999) protocol. Primer characterization and detailed procedures can be seen in Borges et al., (Chapter 1). Genotyping was performed following the Creste et al., (2001) protocol for the silver-stained 6% denaturing polyacrylamide gel technique, with the aid of a 10pb ladder to allow for accurate determination of band size.

Because of the multiband pattern observed for this species and the ensuing difficulty in assessing allelic dosage, we treated microsatellite markers as multilocus fingerprints, which were in turn used to build a binary matrix based on the presence/absence of each allele. We then performed an UPGMA analysis of Jaccard's distance, whose complement was used to estimate genetic similarity among ramets and check for multiclonal clumps utilizing the r package vegan v.2.4 (OKSANEN et al., 2017). The total number of individual genotypes divided by the samples number, the proportion of distinguishable genets (PD) was calculated as a descriptor of the genetic diversity within the clumps (KREHER et al., 2000). We also counted the number of band differences among culms of the same clump for each microsatellite locus.

Results

Genetic distance analysis at the culm level revealed 34 multilocus genotypes, which is a depart from what would be expected if all culms within a clump were in fact genetically identical. Of the 17 multi-ramet clumps, 12 contained at least two different genotypes. The number of genotypes within multiclonal clumps ranged from two to four (Table 1). Still, in all but one case, non-identical ramets belonging to the same clump were much genetically closer among themselves than they were to ramets of adjacent clumps (Figure 1). The proportion of distinguishable genets (PD) was 0.46, and the number of band differences within multiclonal clumps ranged from one to five.

UPGMA tree based on Jaccard's distance also revealed the existence of two bigger genetic groups. These groups match the environment-based genetic clusters found in previous studies with the same population (Chapter 2).

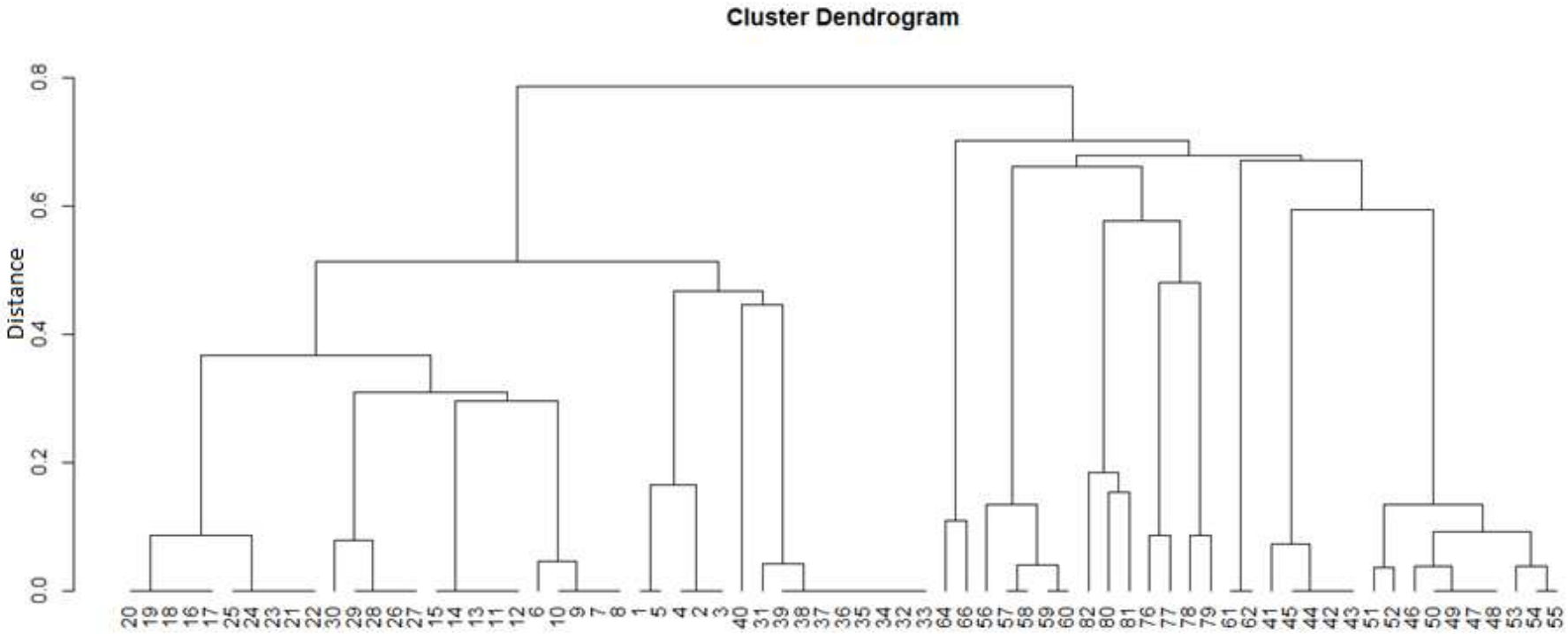

Figure 1 UPGMA tree based on Jaccard's distance, showing the coexistence of multiple genotypes within some clumps, as well as the close relationship among non-identical ramets of the same clump (identified by ID numbers; see Table 1 for further references on culm IDs).

Table 1 Number of observed *M. neesii* genets and mean number of band differences per SSR locus.

Sample IDs	**Clump size (n° of culms)**	**Nº of sampled culms**	**Nº genotypes**	**Mean number of band differences**
1-5	> 10	5	2	1.3
6-10	> 10	5	2	1
11-15	> 10	5	1	0
16-20	> 10	5	1	0
21-25	> 10	5	1	0
26-30	> 10	5	2	2
31-35	> 10	5	2	1
36-40	> 10	5	2	1
41-45	> 10	5	2	1
46-50	> 10	5	2	1
51-55	> 10	5	4	1.5
56-60	> 10	5	3	2
61-62	3	2	1	0
64-66	5	3	2	2
76-77	4	2	2	2
78-79	2	2	2	2
80-82	6	3	3	5

Discussion

This population of *M. neesii* displays a high level of genetic variation within clumps (PD value 0.46) much higher than the average of 0.17 calculated for 27 clonal species using allozyme markers (ELLSTRAND & ROOSE, 1987). However, more recent studies using nuclear markers, such as RAPD and SSR, have revealed overall higher values of PD (CHEN et al., 2006; HONG & LEe, 2015). KREHER et al., (2000) suggest a possible relationship between the mode of clonal spread and the number of genets observed in a population, such that rhizome propagated species tend to show higher PD values than species that spread by other means such as severed twigs and buds.

Multiple mechanisms have been suggested to explain the existence of several genotypes within discrete clumps or patches (TORIMARU & TOMARU, 2005). Somatic mutations have been showed to be an important force promoting genetic diversity in clonal plants (SÁNCHEZ-VILAS et al., 2010). Fernando & Cass (1996) proposed that single band differences between the genetic profiles of *Butomus umbellatus* could have been due to somatic mutations. O'Connell & Ritland (2004) also showed that single-band differences between genotypes based on microsatellite markers were due to somatic mutation. In seven of the 13 multiclonal clumps, culms differed among themselves by more than one band. While microsatellite mutation rates are considerably higher than in coding regions, they still range between 10^{-2} to 10^{-6} events per locus per generation (LIAN et al., 2004). As such, it seems highly unlikely that somatic mutations could account for such amount of variation within the clumps (KREHER et al., 2000).

Furthermore, assuming that slipped-strand mispairing is the main cause of changes in microsatellite length – and thus, that single-step mutations are considerably more frequent than changes involving two or more steps –, if somatic mutations were in fact responsible for inter-ramet variation in *M. neesii*, we would expect to see a pattern of mostly small variations in the number of microsatellite repeats among culms of the same clump (BRUVO et al., 2004; LIAN et al., 2004). Instead, we noted that, among non-identical culms belonging to the same clump, band size differences were such that would require several steps of mutation.

The abundant clonal diversity within *M. neesii* clumps may also be explained by seedling recruitment patterns (TORIMARU & TOMARU, 2005). This might have taken

the form of several founding events occurring within strict spatial limits, with genets originating from seeds arriving and establishing in several distinct moments over time (PADGURSCHI, 2014). On the other hand, as evidenced by genetic distance data, culms that belong to the same clump are more related to each other than to culms in other clumps. This suggests that either clump founders were systematically related to each other, or, more likely, that seedling recruits did not migrate far from the mother plant, and what we experience as a discrete clump is, in fact, a cluster of siblings that established themselves around the same time (KREHER et al., 2000).

While the timing of seed recruitment in *M. neesii* is not yet entirely clear, the latter hypothesis is further supported by the fact that, as many bamboos, *M. neesii* seeds lack adaptations to dispersal – which, in turn, gives rise to a distinct pattern of isolation-by-distance. The closer two genets are in space, the more closely related they tend to be (ORSINI et al., 2013). This pattern was confirmed through a genetic analysis performed at the clump level, in which kinship values are highest below 3m of distance (Chapter 2). It is possible that the isolation-by-distance pattern extrapolates into the genet level: if genetic and geographic distances are directly correlated, what greater closeness is there than sibling genets occupying the same clump?

The finding that some bamboo clumps are constituted of several closely-related genets also resonates with literature regarding the evolution of this group's distinct life cycle. It has been proposed by some authors that the death of reproductive individuals following seed setting improves seedling survival rates by causing the opening of gaps in the canopy – which allows the entrance of sunlight -, and by providing nutrients in the form of litter (NICHOLSON, 1922; PADGURSCHI, 2014). However, according to Janzen (1976), this would require limited dispersion of seeds, otherwise the individual parent would be dying to open a site for the offspring of other individuals. Given that genets belonging to the same clump were found to be very closely related – possibly originating from the same mother –, the gap-opening strategy may constitute an evolutionary strategy akin to kin selection, where the mother plant dies to improve the survival odds of its offspring (FILE et al., 2012).

In this sense, bamboo clumps combine both sexual and assexual reproduction in order to succesfully colonize large portions of the forest. Massive – albeit infrequent – sexual events assure the maintainance of high levels of genetic diversity, which in turn contributes to the species' adaptability (FRANKHAM, 2005). At the same time, the

gap-opening strategy assures adequate sites for seedling establishment. Subsequent clonal propagation, in its turn, further contributes to the colonising prowess of this species. All of these traits may explain the predominance of bamboo stands in large portions of the forest.

In summary, this research has revealed that there can be genetic diversity inside *M. neesii* clumps and that clumps do not always equal one genotype. It also showed that the most likely mechanism that drove the appearance of multiclonal clumps is the limited dispersion of seeds, which results in siblings establishing close to each other along favorable patches created by the death of their mother plant. Nevertheless, more thorough genetic and ecological analyses are necessary to clarify the roles of both somatic mutations and multiple founding events in the formation of multiclonal clumps, as well as to further explore the connection between this phenomenon and the evolution of certain reproductive strategies. The knowledge generated in the present study, as well as future developments on the subject, may help conservation policy-makers make informed decisions on how to manage bamboo populations – either to minimize its negative impact on some communities, or simply to preserve a much important element of the forest's ecological balance.

DISCUSSÃO

A limitação na dispersão dos propágulos resulta, conforme previsto pelo modelo clássico de *Isolamento por Distância*, em uma acentuada estrutura genética espacial em fina escala; i.e., genótipos aparentados tendem a permanecer próximos uns dos outros. Por outro lado, as análises também revelaram a existência de duas subpopulações com quase nenhum fluxo gênico entre si, cujo arranjo espacial não pode ser explicado somente pela dispersão limitada dos propágulos. A existência desses agrupamentos genéticos distintos provavelmente está ligada ao padrão de distribuição diferenciada de condições e recursos ao longo da paisagem, um fenômeno que, por sua vez, está relacionado ao alto grau de heterogeneidade ambiental típico de florestas tropicais e ao padrão microtopográfico irregular do local onde a população está estabelecida. Estudos mais aprofundados são necessários para estabelecer exatamente quais condições ambientais estão por trás do estabelecimento diferenciado de genótipos no espaço e da consequente limitação do fluxo gênico entre as subpopulações de *M. neesii*. As análises também foram eficientes em determinar o nível de identidade clonal dentro das moitas. Nossos resultados evidenciam que os colmos dentro de uma mesma moita discreta não são necessariamente clones oriundos do mesmo zigoto (rametas); ao contrário, estes podem diferir entre si e vir a formar moitas *multiclonais*. No entanto, colmos não-idênticos que integram a mesma moita multiclonal demonstram ser significativamente mais aparentados entre si do que em relação a colmos de moitas vizinhas. Esse padrão também pode estar ligado à limitação na dispersão das sementes e, em última instância, ser reflexo de uma estratégia evolutiva mais ampla relacionada ao ciclo de vida próprio dos bambus. Estudos futuros se fazem necessários para testar essa última hipótese, bem como esclarecer o papel de outros mecanismos geradores de variabilidade genética no surgimento da multiclonalidade.

CONCLUSÃO

Os marcadores de microssatélites, bem como as análises estatísticas empregadas, foram eficientes em sua tarefa de revelar a existência de estrutura genética espacial em diversas escalas nesta população do bambu *Merostachys neesii*. Os processos ligados à geração de estrutura genética espacial repercutem, inclusive, na percepção da individualidade na espécie. Estudos posteriores se fazem necessarios para confirmar algumas das hipoteses levantadas no presente trabalho.

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ANEXOS

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DECLARAÇÃO

Em observância ao **§5° do Artigo 1° da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Dissertação de Mestrado, intitulada "***Estrutura genética espacial de uma população de bambus em Hotspot de biodiversidade***", desenvolvida no Programa de Pós-Graduação em Biologia Vegetal do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **"Estrutura genética espacial de uma população de bambus em hotspot de biodiversidade"**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

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